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Sequencing, chromosomal mapping, and functional characterization of bovine FLICE-Like Inhibitory Protein *(FLIP)*

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Abstract. FLICE-like inhibitory protein (FLIP) has been shown in both humans and mice to inhibit apoptosis and NF-κB activation induced by pro-inflammatory mediators. The activation of NF-κB and the induction of apoptosis are critical events in the pathogenesis of a variety of disease states in cattle, including mastitis. Since FLIP is known to moderate these events in other species, we mapped the bovine FLIP gene, sequenced bovine *FLIP* cDNA, and characterized its expression in cultured primary bovine endothelial cells. Sequencing of bovine *FLIP* revealed approximately 83, 74, and 68 % amino acid sequence identity to its porcine, human, and murine orthologs, respectively. Bovine *FLIP* was mapped to chromosome 2 by radiation hybrid mapping. Interestingly the region to which bovine *FLIP* maps contains a putative quantitative trait locus for functional herd life which is an indicator of a cow's ability to survive involuntary culling due primarily to mastitis and infertility. In addition to sequencing and mapping, the function of bovine FLIP was studied. Over-expression of bovine FLIP protected against bacterial lipopolysaccharide (LPS)- and TNF-α-induced apoptosis in bovine endothelial cells consistent with previous studies of human FLIP. In addition, elevated expression of bovine FLIP blocked LPS- and TNF-α-induced upregulation of NF-κB-dependent gene products as assayed by E-selectin expression. Only the full-length bovine FLIP protein could inhibit NF-κB activation induced by LPS, whereas the death effector domain region alone was able to inhibit TNF-α-induced NF-κB activation. Together, these data demonstrate the conservation of FLIP's ability to inhibit apoptosis and to downregulate NF-κB activation across species.

Bacterial infections are responsible for many of the most economically important diseases in cattle including mastitis (Wells et al., 1998). Mastitis is an inflammatory disease that develops in response to intramammary infection (Sordillo et al., 1997; Burvenich et al., 2003). Controlled inflammation within the gland benefits the host by limiting the growth and spread of harmful bacterial pathogens. Excessive inflammation, however, can lead to injury of host tissue and the development of systemic complications resulting in septic shock. The excessive inflammation and injury that often develop are caused by both bacterial cell wall components and host-derived mediators, including bacterial lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α, respectively (Ohtsuka et al., 1997, 2001; Burvenich et al., 2003). Both LPS and TNF-α are capable of activating NF-κB, a transcription factor that induces the expression of cytokines and adhesion molecules that contribute to the inflammatory response (Tak and Firestein, 2001). In addition to activating cells, LPS and TNF-α are potent inducers of apoptosis in a variety of cell types (Harlan et al.,

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1983; Madge and Pober, 2001; Bannerman and Goldblum, 2003).

The vascular endothelium is a key host target of LPS and TNF-α (Olson et al., 1995; Madge and Pober, 2001; Bannerman and Goldblum, 2003). Endothelial cells (EC), which line the vasculature, play a critical role in inflammation by regulating leukocyte trafficking and vascular permeability (Cines et al., 1998; Stefanec, 2000). EC express receptors for both LPS and TNF-α and by virtue of their location at the blood-tissue interface are one of the first cell types to encounter circulating levels of either of these pro-inflammatory mediators. The pro-inflammatory effects of LPS and TNF-α are due, in part, to their ability to elicit several NF-κB-dependent EC responses including: 1) the production of the pro-inflammatory cytokines, IL-1_β, IL-6, and IL-8, 2) the surface expression of the adhesion molecules, E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, and 3) the expression of tissue factor (Olson et al., 1995; Bierhaus et al., 2000; Madge and Pober, 2001). In addition to activation, LPS and TNF-α induce EC apoptosis (Pohlman and Harlan, 1989; Zen et al., 1999), an event which is believed to contribute to the pathogenesis of sepsis and its attendant complications (Haimovitz-Friedman et al., 1997; Stefanec, 2000). Interestingly, EC derived from cattle are exquisitely sensitive to the injurious effects of LPS and TNF-α relative to those of other species, however, the mechanism for this differential sensitivity remains unknown (Harlan et al., 1983; Brigham and Meyrick, 1986; Meyrick et al., 1986; Robaye et al., 1991; Bannerman and Goldblum, 2003).

FLICE-like inhibitory protein (FLIP) has been established to protect against LPS-induced apoptosis in human cells (Bannerman et al., 2004) and TNF-α-induced apoptosis in cells derived from humans and mice (Yeh et al., 2000; Okada et al., 2001). FLIP shares significant homology with caspase-8, a protease whose activation initiates the onset of apoptosis (Irmler et al., 1997). A two amino acid substitution in the region of FLIP corresponding to the catalytic active site of caspase-8 renders FLIP incapable of proteolysis. Thus, FLIP functions as a natural dominant-negative inhibitor of caspase-8. Relative changes in the expression of FLIP and caspase-8 can dictate whether a cell will survive or undergo apoptosis in response to a given stimulus (Tepper and Seldin, 1999; Fulda et al., 2000). Overexpression of human FLIP protects against LPS-induced EC apoptosis, whereas targeted downregulation of FLIP enhances sensitivity to the pro-apoptotic effects of LPS (Bannerman et al., 2004). In addition to its role in mediating apoptosis, FLIP has recently been shown to downregulate NF-κB activation (Wajant et al., 2000; Bannerman et al., 2004). Thus, human FLIP is an established modulator of LPS-induced pro-inflammatory and pro-apoptotic signaling. Because of the exquisite sensitivity of bovine EC to the pro-inflammatory and injurious effects of LPS and TNF-α, we investigated whether bovine FLIP, similar to its human homologue, can block apoptosis and inhibit NF-κB dependent gene expression elicited by these agonists.

Materials and methods

Materials

Highly purified LPS, phenol extracted from *Escherichia coli* serotype 0111:B4 and further purified by ion exchange chromatography was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human and murine TNF-α were obtained from R&D Systems, Inc. (Minneapolis, MN). Cycloheximide (CHX), which was used at a final concentration of 40 μg/ml to sensitize EC to TNF-α-induced apoptosis, was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA).

Cell culture

Bovine mammary secretory epithelial cells (generous gift of Dr. A.J. Guidry, USDA-ARS, Beltsville Agricultural Research Center, Beltsville, MD) were cultured in 1:1 RPMI-1640 and DMEM (Cambrex Corp., Walkersville, MD) enriched with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), insulin (5 μg/ml), hydrocortisone (1 μg/ml) (Sigma Chemical Co.), HEPES (40 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Cambrex Corp.) as previously described (Cifrian et al., 1994). Bovine aortic, pulmonary artery, and mammary artery EC (generous gifts of Dr. L.M. Sordillo, Michigan State University, East Lansing, MI and Dr. A.J. Guidry, USDA-ARS, Beltsville Agricultural Research Center, Beltsville, MD) were cultured in Ham's F12K medium (Irvine Scientific Sales Co., Santa Ana, CA) supplemented with 10% fetal bovine serum, heparin (100 μg/ml), insulin (10 μg/ml), transferrin (5 μg/ml) (Sigma Chemical Co.), HEPES (20 mM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The human microvascular endothelial cell (HMEC)-1 line (generously provided by F.J. Candal and Dr. E. Ades, Centers for Disease Control, and Dr. T. Lawley, Emory University, Atlanta, GA) was cultured as previously described (Bannerman et al., 2004). *Flip*$^{+/-}$ and *Flip*$^{-/-}$ mouse embryo fibroblasts (MEF) (generous gift of Dr. Wen-Chen Yeh, Amgen Institute, Toronto, Canada) were generated as previously described (Yeh et al., 2000) and cultured in DMEM medium enriched with 10% fetal bovine serum, L-glutamine (2 mM), sodium pyruvate (1 mM), and nonessential amino acids, in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured at 37 °C in the presence of 5% CO_2.

RNA isolation

Cells were grown to confluence, rinsed twice with PBS, detached with trypsin, and centrifuged at 220 *g* for 5 min at 4 °C. The cell pellet was rinsed twice with PBS and cytoplasmic RNA isolated using the Rneasy Mini Kit (Qiagen, Inc., Valencia, CA). Prior to the final elution from the column, mRNA was subjected to DNase digestion with 340 Kunitz U/ml of DNase I (Qiagen, Inc.) for 15 min at room temperature. Concentrations and quality were initially determined by OD (A260/280) and agarose gel electrophoresis and confirmed with an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

Cloning and sequencing

For cloning and sequencing of bovine *FLIP*, cytoplasmic RNA from bovine mammary secretory epithelium was used as template for 5′ and 3′ rapid amplification of cDNA ends (RACE). First-strand cDNA was obtained using the GeneRacer Kit (Invitrogen Corp., Carlsbad, CA) and an oligo d(T) primer according to manufacturer's instructions. The initial primers used in 5′ and 3′ RACE were 5′ TCC CAG GGG CTT GCT CTG CAT CTT A 3′ and 5′ GCC CTC CAG CTC ACC CTC ATT GT 3′, respectively. Primers were designed from well conserved regions of human (NM_003879) and murine (NM_009805) *FLIP* mRNA. RACE reactions were performed in a 50-μl reaction volume as per kit instructions using the following cycling conditions: 94 °C for 2 min; 5 cycles of 94 °C for 30 s, 72 °C for 1 min; 5 cycles of 94 °C for 30 s, 70 °C for 1 min; 25 cycles of 94 °C for 30 s, 65 °C for 30 s, 68 °C for 1 min; and a final extension cycle of 68 °C for 10 min. RACE products were cloned into a plasmid vector using the TOPO cloning kit for sequencing (Invitrogen) and inserts sequenced in both directions using vector- and gene-specific primers. All sequencing was performed using a CEQ8000 automated DNA sequencer and Quickstart Chemistry (Beckman Coulter, Fullerton, CA). Subsequent RACE reactions were carried out to gain additional sequence information using primers based on the results of earlier RACE reactions. This strategy determined the nucleotide sequence of the 3′ end of the transcript, but failed to determine the start of the coding region. To

obtain 5′ end sequence, first-strand cDNA was generated from mammary secretory epithelial cytoplasmic RNA using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions for preparation of 5′ RACE-ready cDNA. Subsequent PCR was performed using Advantage 2 Polymerase Mix (BD Biosciences Corp., Franklin Lakes, NJ) and the following primers: (1) 5′ TTT CTT CCT GCC ATT CCT TG 3′, which corresponds to the 5′-UTR of the alternatively spliced bovine *FLIP* short form and (2) 5′ TGT TGA CTT TTT ATT TGT GAG AGA GG 3′, which corresponds to bovine *FLIP* sequence obtained by 3′ RACE (underlined nucleotides of the latter primer correspond to the published bovine *FLIP* sequence). Cycling conditions were: 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 53.5 °C for 30 s, 68 °C for 2 min; followed by 68 °C for 10 min. The resulting 1,502-bp product was cloned into a plasmid vector (TOPO TA Cloning Kit, Invitrogen) and sequenced as described above.

Mapping

Chromosomal mapping of bovine *FLIP* was carried out using a 3000-rad bovine/hamster radiation hybrid (RH) panel (ResGen Invitrogen, Carlsbad, CA). DNA from a panel of 94 clone lines was screened by PCR using primers (5′ GGA TCT TGT GGT TGA ATT GGA 3′ and 5′ AAT TTG TTT CCG CTC CTT GA 3′) designed to amplify an ~ 1,100-bp fragment spanning putative intron 4 of *FLIP*, based on human gene structure information (http://www.ncbi.nlm.nih.gov/LocusLink/). Amplification conditions were as follows: 94 °C for 30 s; 10 cycles of 65 °C for 30 s (–1°/cycle) and 68 °C for 2 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min; and a final extension of 68 °C for 10 min. Products were separated by agarose gel electrophoresis and partial sequencing of the amplicon performed to confirm identity as bovine *FLIP*. Concordancy data for each cell line were submitted to the Roslin RH database (http://databases.roslin.ac.uk/radhyb/intro.py) for mapping as previously described (Connor et al., 2004).

Quantitative RT-PCR

Abundance of *FLIP* transcripts and transcripts for the housekeeping genes ATP synthase *(ATP5B)* and hydroxymethylbilane synthase *(HMBS)* were measured by quantitative real-time PCR using an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). First strand cDNA was synthesized from 500 ng of cytoplasmic RNA of each cell line of interest with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) in a 20-µl reaction volume, according to manufacturer's instructions. Negative controls (minus reverse transcriptase) were also synthesized for each sample to ensure the absence of contaminating genomic DNA. Subsequent PCR was performed in duplicate (single reactions for negative controls) using 2 µl of first strand cDNA in a 25-ml reaction volume, 1× iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.1 µM of each primer. Primer pairs were designed for each species for amplification of *FLIP*. Each pair (5′ GAA CCA GTG AAG AGA CCC ATT C 3′ and 5′ GAA GGT GTC CCG AAG AAT GTC 3′ [bovine] or 5′ GAA CCA GTG AAG AAA TCC ATT C 3′ and 5′ GAA GGT GTC TCG AAG AAG CTC 3′ [human]) amplifies a 150-bp fragment corresponding to a region unique to the long isoform of *FLIP*. Cycling conditions were 95 °C for 3 min followed by 45 cycles of 94 °C for 15 s, 55.7 °C for 30 s and 72 °C for 30 s with fluorescence measurement occurring during the extension step. For *ATP5B* and *HMBS*, universal primer pairs were designed to amplify 148- and 127-bp products, respectively, from both species. Primers for *ATP5B* were 5′ CAT CGT GGC GGT CAT TGG 3′ and 5′ AAT GGT CCT TAC TGT GCT CTC 3′ and for *HMBS* were 5′ TGC TTC CTC CTG GCT TCA C 3′ and 5′ GTT CCT ACC ACA CTC TTC TCT G 3′). Cycling conditions were the same as for *FLIP* except that the annealing step was done at 58.2 °C. External standard calibration curves were generated for all assays from known quantities of purified double-stranded DNA containing each region of interest. Standards ranging from 102–106 molecules based on a single-stranded DNA molecule calculation were analyzed in duplicate for each assay. Efficiencies of amplification were calculated by the iCycler iQ Software Version 3.0A using the formula $E = 10^{[-1/slope]} - 1$.

Cloning and stable expression of cDNA constructs

To overexpress full-length or truncated constructs of bovine *FLIP* containing a FLAG tag (DYKDDDDK), PCR was used to generate products with 5′ *Eco*RI (GAA TTC) and 3′ *Xho*I (CTC GAG) restriction sites for insertion into the pCR3.V64 Met FLAG vector. Template for PCR was the cloned coding region of bovine *FLIP*. The primers used to generate the various constructs, with gene specific regions indicated by an underscore, are as follows: 1) 5′ CCG GAA TTC ATG TCT GCT GAA GTC ATC CAT 3′ and 5′ CCG CTC GAG TTA TTT GTG AGA GAG GAA GAG 3′ for the generation of full-length FLIP (AA 1–484); 2) 5′ CCG GAA TTC ATG TCT GCT GAA GTC ATC CAT 3′ and 5′ CCG CTC GAG TTA CCT TAG GTT ATA TGA AGG ATC 3′ for the generation of the FLIP death effector domain (DED) (AA 1–203); and 3) 5′ CCG GAA TTC TTG CCT CAG CAC ATA GTA GAA 3′ and 5′ CCG CTC GAG TTA TTT GTG AGA GAG GAA GAG 3′ for the generation of the FLIP caspase domain (AA 237–484). Cycling conditions were as follows: 94 °C for 1 min, then 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min, followed by 10 min at 68 °C. The PCR products were gel-purified, digested with *Eco*RI and *Xho*I, and ligated into the pCR3.V64 Met FLAG vector. This vector contains a 5′ *Bam*HI site followed by a coding region for an initiator methionine, the FLAG tag, and a spacer of two amino acids (EF) immediately before the *Eco*RI – *Xho*I PCR product insertion site. Bacteria were transformed with the ligation reactions and clones from bacteria containing appropriate sized inserts were fully sequenced. Correct clones were then subcloned into the *Bam*HI and *Xho*I restriction sites of the pBMN-IRES-PURO retroviral expression vector (kindly provided by Dr. Gary Nolan, Stanford University, Stanford CA and subsequently modified by Dr. Kyle Garton, University of Washington, Seattle, WA).

High-titer retrovirus was prepared from the Phoenix amphotropic packaging cell line (ATCC, Manassas, VA) transfected with 24 µg of the expression plasmid by calcium phosphate precipitation. Recombinant retroviral supernatants were collected 48 h after transfection and filtered through a Millex-HV 0.45-µm filter (Millipore Corp., Bedford, MA). For infection, EC or MEF were seeded into 6-well plates and grown until reaching ~ 80 % confluence. The growth medium was replaced with 2.5 ml of retroviral supernatant supplemented with 8 µg/ml polybrene and 10 mM HEPES, and the plate centrifuged for 1.5 h (1,430 *g*; 32 °C). An additional 2.5 ml of growth medium were added to each well and the cells were then incubated for 8 h (5 % CO_2, 37 °C), after which the retroviral-containing supernatant was replaced with normal growth medium. Twenty-four hours post-infection, growth medium was supplemented with 2 µg/ml puromycin (Sigma Chemical Co.) to select for cells stably expressing the construct of interest. Flow cytometry demonstrated that ~ 99 % of the cells infected with the control vector encoding enhanced green fluorescent protein (EGFP) were positive for EGFP expression following puromycin selection (data not shown). Western blotting was performed as previously described (Bannerman et al., 2004) with 1:2,000 diluted anti-FLAG antibody conjugated to horseradish peroxidase (Sigma Chemical Co.) to confirm expression of the constructs.

Caspase assay

Cells were seeded into 96-well plates and cultured until confluent. Following treatment, caspase activity was measured with a fluorimetric homogeneous caspases assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). The plates were analyzed on a fluorescence plate reader (Bio-Tec Instruments, Inc., Winooski, VT) at a 485-nm excitation and a 530-nm emission, and caspase activity expressed relative to a simultaneous medium control.

E-Selectin ELISA

EC were seeded into 96-well plates and cultured until confluent. Following treatment, cells were washed twice with RPMI-1640 medium supplemented with 2.5 % bovine calf serum and fixed for 10 min with 0.5 % glutaraldehyde in PBS at room temperature. Monolayers were washed and incubated for 1 h at 37 °C with anti-E-selectin (CD-62E) rabbit polyclonal antibodies (NeoMarkers, Inc., Fremont, CA) diluted 1:1,000 in wash buffer. The cells were washed twice and incubated as above with horseradish peroxidase-conjugated goat anti-rabbit antibodies (BD Biosciences Corp.) diluted 1:2,000 in wash buffer. The wells were then washed five times with PBS and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added to each well. The reaction was stopped by the addition of 100 µl of 2 M H_2SO_4 and the absorbance read at 450 nm on a microplate reader (Bio-Tec Instruments, Inc.). A background correction reading at 565 nm was subtracted from the 450 nm absorbance readings.

Statistical methods

A t test or analysis of variance (ANOVA) was used to compare the mean responses between a single experimental group or multiple experimental

groups, respectively, and the control group. For experiments analyzed by ANOVA, the Tukey post hoc comparison test was used to determine between which groups significant differences existed. All statistical analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, Inc., San Diego, CA). A P value of <0.05 was considered significant.

Results and discussion

From three clones of bovine *FLIP* cDNA obtained by RT-PCR, a consensus sequence of 1,496 bases was obtained (GenBank accession no. AY882619) sharing 87, 85 and 83% identity to porcine (NM_001001628), human (NM_003879), and murine (NM_207653) *FLIP* mRNA, respectively. Using a 3000-rad bovine/hamster radiation hybrid panel, *FLIP* was mapped to bovine chromosome 2 (BTA2) near marker BMS2626 (LOD = 7.19, distance = 0.361 cR). This region corresponds to approximately 70 cM on the USDA linkage map (http://www.marc.usda.gov/genome/). Mapping of *FLIP* to BTA2 is consistent with the predicted location based on current human-bovine comparative maps (Hayes et al., 2003). Of interest, the region of BTA2 to which bovine *FLIP* maps contains a putative quantitative trait locus (QTL) for functional herd life in Holstein cattle (Kuhn et al., 2003). This longevity trait is a measure of a cow's ability to survive involuntary culling, the primary causes for which are mastitis and infertility. Thus, *FLIP* is a positional candidate gene contributing to the functional herd life trait.

FLIP expression was determined by quantitative RT-PCR in bovine EC derived from the aorta (BAEC), mammary artery (BMEC), and pulmonary artery (BPAEC) (Table 1). Efficiency of amplification for all assays ranged from 82 to 95% and correlation coefficients for all standard curves were >0.997. Transcript abundance in all negative controls was below the detection limit of the assay. Expression levels were expressed relative to either total RNA or to the number of molecules of the housekeeping genes ATP synthase *(ATP5B)* and hydroxymethylbilane synthase *(HMBS)*. The expression levels of *FLIP* among the different bovine cell types were within a one-fold difference of one another. In contrast, the expression level of *FLIP* in EC derived from humans (HMEC) was ~7–19 times greater than that of bovine, depending on the method of standardization. We have previously shown that HMEC are resistant to the direct effects of LPS-induced apoptosis and that targeted down-regulation of *FLIP* sensitizes these cells to LPS-evoked apoptosis (Bannerman et al., 2004). In contrast, the three different bovine EC types tested, all of which had lower FLIP levels than HMEC, display significantly higher levels of caspase activity following LPS exposure than HMEC (Table 2). Thus, the enhanced sensitivity of bovine EC to LPS-induced apoptosis may be due to lower expression of *FLIP*. Alternatively, the bovine FLIP protein may be non-functional and unable to inhibit LPS-induced apoptosis.

The bovine *FLIP* cDNA encodes a predicted 484-amino-acid protein sharing 83, 74, and 68% amino acid sequence identity with porcine, human, and murine sequences, respectively (Fig. 1). Similar to its orthologs in human, mice, and pigs, bovine *FLIP* encodes two DED (aa 2–79 and 93–176) and a caspase domain (254–482). The DED regions are protein-protein binding domains that facilitate FLIP recruitment to death receptor complexes localized to the cell membrane (Tschopp et al., 1998; Krueger et al., 2001). The role of FLIP in the inhibition of death receptor signaling such as that activated by Fas or TNF-α, has been well elucidated. Upon Fas or TNF-α binding to its cognate receptor, a multi-protein complex is assembled that leads to the recruitment of the adapter protein FADD via its conserved death domains (DD) (Natoli et al., 1998; Peter and Krammer, 1998). FADD, in turn, can recruit FLIP and/or pro-caspase-8 via protein-protein interactions of DEDs contained within all three proteins. Pro-caspase-8 has intrinsically low levels of proteolytic activity which enables it to cleave other pro-caspase-8 proteins brought into close proximity following their recruitment to FADD (Muzio et al., 1998). Activation of caspase-8 initiates a proteolytic cascade resulting in the activation of downstream effector caspases. One mechanism by which FLIP is proposed to inhibit apoptosis is by competitively binding to FADD via respective DED-DED interactions and blocking recruitment of pro-caspase-8 (Thome et al., 1997; Tepper and Seldin, 1999). Thus, the DEDs are critical to the ability of FLIP to function as an anti-apoptotic protein. Within both DED regions of FLIP, amino acid sequence identities were ~85% with the corresponding regions in human FLIP, indicating high conservation of these critical regions.

Another mechanism by which FLIP is postulated to inhibit apoptosis is via its inability to activate pro-caspase-8 (Krueger et al., 2001). In this model, death receptor activation leads to FADD recruitment of both pro-caspase-8 and FLIP, however, the lack of intrinsic FLIP proteolytic activity prevents the

Table 1. *FLIP* mRNA expression in endothelial cells derived from various tissues

Cell type	*FLIP* (molecules/ng RNA)		*FLIP* (molecules/*ATP5B*)[a]		*FLIP* (molecules/*HMBS*)[a]	
BAEC	134	(1.00)[b]	0.017	(1.00)	0.284	(1.00)
BMEC	294	(2.19)	0.027	(1.58)	0.426	(1.50)
BPAEC	98	(0.73)	0.012	(0.71)	0.264	(0.93)
HMEC	942	(7.03)	0.274	(16.12)	5.358	(18.87)

[a] Ratio of *FLIP* molecules to the number of molecules of the housekeeping genes ATP synthase (*ATP5B*) and hydroxymethylbilane synthase (*HMBS*), respectively.
[b] Values in parentheses indicate mRNA expression relative to BAEC.

Table 2. LPS-induced caspase activity in endothelial cells derived from various tissues

Cell type	Caspase activity[a]	S.E.M.	n
BAEC	15.70[b]	1.785	5
BMEC	11.06[b]	0.926	5
BPAEC	13.65[b]	0.722	5
HMEC	1.35	0.070	6

[a] Caspase activity in endothelial cells exposed to LPS (100 ng/ml) for 16 h and expressed relative to EC treated with medium alone.
[b] Significantly increased relative to HMEC ($P < 0.01$).

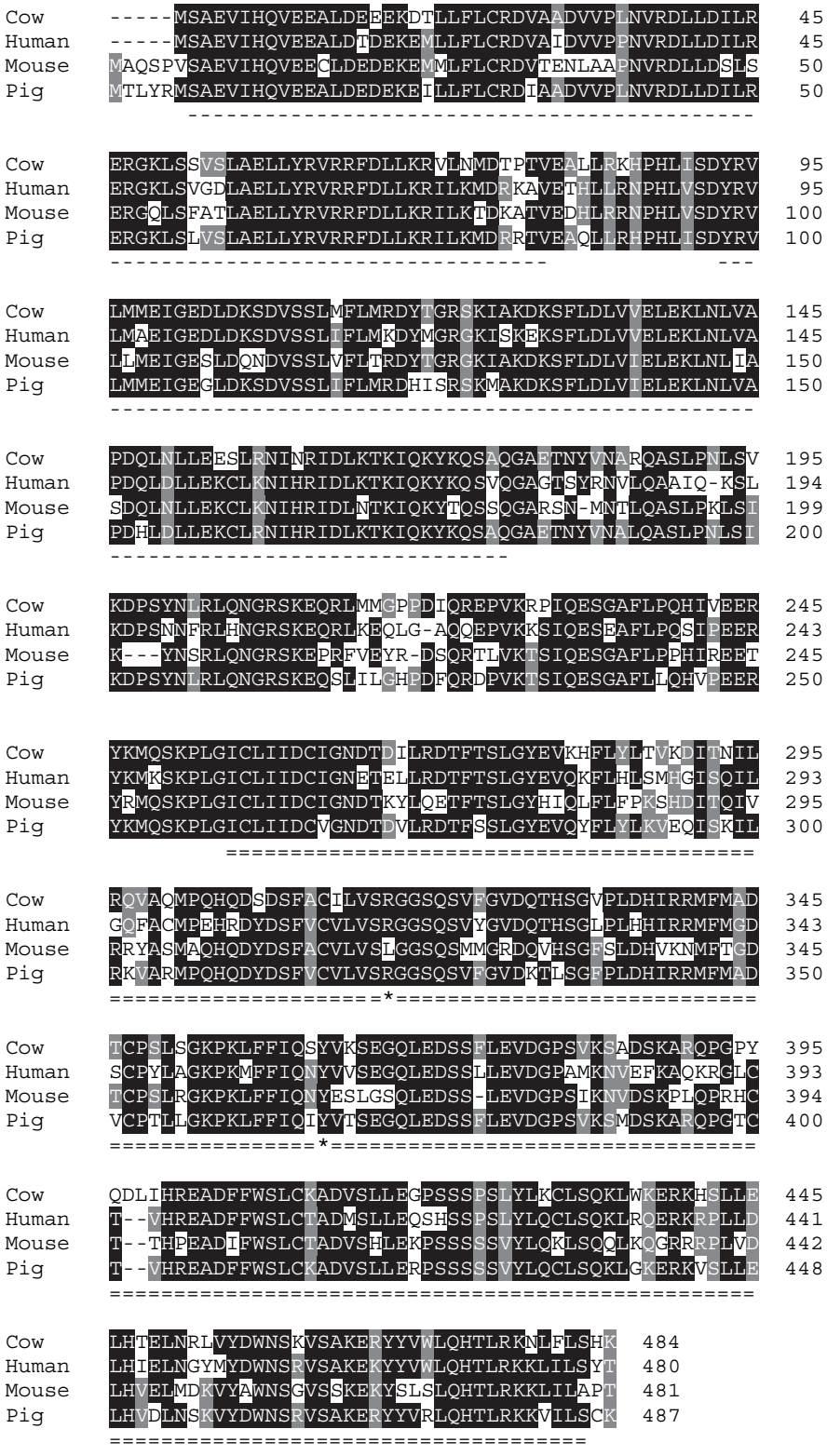

Fig. 1. Amino acid sequence alignment of bovine, human, murine, and porcine FLIP. Black-shaded boxes indicate conservation of an individual amino acid across three or more of the FLIP homologues, whereas, grey-shaded boxes indicate conservation among two of the FLIP homologues. Single and double dashed lines denote the two DED regions and one caspase domain, respectively. Asterisks indicate conservation of amino acids in FLIP rendering it catalytically inactive relative to the highly homologous caspase-8 protein, the latter of which possesses proteolytic activity.

transactivation of pro-caspase-8 to a fully functional mature caspase. The caspase-like domain of FLIP is highly homologous with that of caspase-8. However, corresponding histidine and cysteine residues contained within human caspase-8 are substituted with arginine and tyrosine in human FLIP rendering the caspase-domain of the latter catalytically inactive (Irmler et al., 1997). Sequencing of bovine *FLIP* cDNA revealed the conservation of these arginine317 and tyrosine362 amino acids, suggesting that bovine FLIP lacks endogenous caspase activity similar to its human counterpart (Fig. 1). Together, based on

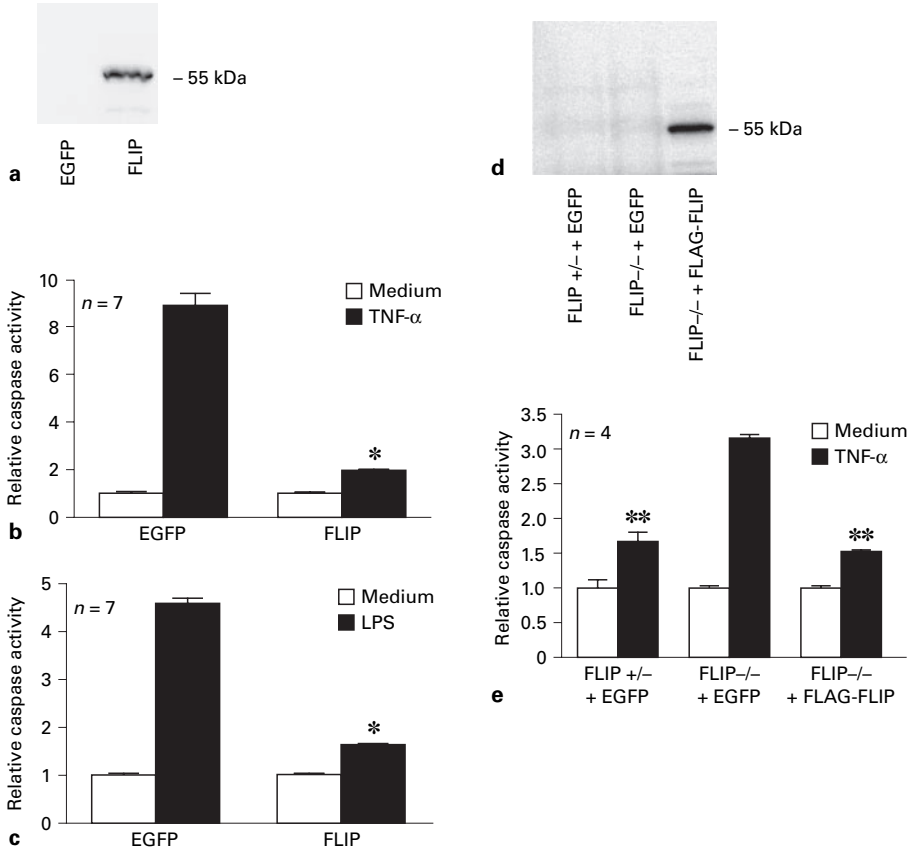

Fig. 2. Effect of bovine FLIP expression on TNF-α- and LPS-induced apoptosis. Bovine aortic endothelial cells (**a**, **b**, **c**) or *Flip*$^{+/-}$ or $^{-/-}$ mouse embryo fibroblasts (**d**, **e**) were stably transfected with either a control vector encoding EGFP or cDNA encoding bovine FLIP. Western blotting with an anti-FLAG antibody was used to confirm expression of the FLIP construct (**a**, **d**). Bovine aortic endothelial cells expressing either EGFP or bovine FLIP were treated with medium, TNF-α (100 ng/ml; 8 h) (**b**), or LPS (100 ng/ml; 12 h) (**c**), and assayed for caspase activity. In other experiments, *Flip*$^{+/-}$ or $^{-/-}$ mouse embryo fibroblasts expressing either EGFP or bovine FLIP were treated with medium 10 ng/ml of murine TNF-α for 12 h, and assayed for caspase activity (**e**). Vertical bars represent mean (+ SE) caspase activity relative to simultaneous medium controls. For each transfected endothelial or fibroblast cell line, the number of treatment replicates is indicated (n). * Significantly decreased compared to EGFP-vector transfected endothelial cells exposed to identical treatment. ** Significantly decreased compared to FLIP$^{-/-}$ fibroblasts exposed to identical treatment.

sequence similarity of the DED regions and the conservation of key amino acids in its caspase-like domain, bovine FLIP is predicted to be a fully-functional anti-apoptotic molecule.

To determine whether bovine FLIP could functionally inhibit apoptosis, a retroviral transfection system was used to overexpress bovine FLIP in EC. Expression was confirmed by quantitative real time PCR (data not shown) and Western blot analysis (Fig. 2a). EC expressing either a control vector encoding EGFP or over-expressing bovine FLIP were treated with 100 ng/ml of TNF-α or LPS for 8 or 12 h, respectively, and assayed for caspase activity (Fig. 2b and c). Relative to EC expressing EGFP alone, those over-expressing FLIP were almost completely resistant to TNF-α- and LPS-induced apoptosis. In subsequent experiments, MEFs from *Flip*$^{+/-}$ and *Flip*$^{-/-}$ mice were retrovirally transfected as above with either an expression vector encoding EGFP or bovine FLIP and treated with medium or murine TNF-α (10 ng/ml) for 12 h. Western blotting, as well as quantitative RT-PCR (data not shown), confirmed expression of bovine FLIP in the respective FLIP$^{-/-}$ MEFs transfected with the bovine FLIP expression vector (Fig. 2d). Similar to a previous report (Yeh et al., 2000), FLIP$^{-/-}$ demonstrated enhanced sensitivity to TNF-α-induced apoptosis as evidenced by increased caspase activity relative to FLIP$^{+/-}$ MEF (Fig. 2e). Reconstitution of FLIP$^{-/-}$ MEF with bovine FLIP completely reversed the enhanced sensitivity to TNF-α-induced apoptosis in the FLIP$^{-/-}$ MEF. Together, these data establish a functional capacity of bovine FLIP to protect against LPS- and TNF-α-induced apoptosis and are consistent with the ability of its human and murine homologues to similarly protect against apoptosis elicited by either of these agonists.

In addition to its role in preventing apoptosis, human FLIP has been reported to downregulate NF-κB, the latter of which regulates the expression of several pro-inflammatory gene products (De Martin et al., 2000). Specifically, elevated levels of human FLIP have been shown to inhibit TNF-α- and LPS-induced NF-κB-dependent gene expression (Wajant et al., 2000; Bannerman et al., 2004). To determine whether bovine FLIP could similarly influence NF-κB, EC over-expressing full-length bovine FLIP were exposed to either 10 ng/ml of TNF-α or 100 ng/ml LPS for 8 h and assayed by ELISA for E-selectin (Fig. 3a and b), the expression of which is upregulated by NF-κB (Tak and Firestein, 2001). TNF-α or LPS treatment resulted in a >5-fold increase in E-selectin expression in control EC. EC expressing elevated levels of bovine FLIP demonstrated ~35 and 45% less expression of E-selectin following TNF-α or LPS exposure, respectively, than similarly treated control EC. Thus, similar to its human homologue, bovine FLIP is capable of downregulating NF-κB-dependent gene expression.

To determine whether the entire FLIP molecule or one of its two functional domains alone, the DED or caspase regions, were required for its inhibitory action on NF-κB-dependent gene expression, EC were transfected with expression vectors encoding either the DED or caspase region of bovine FLIP

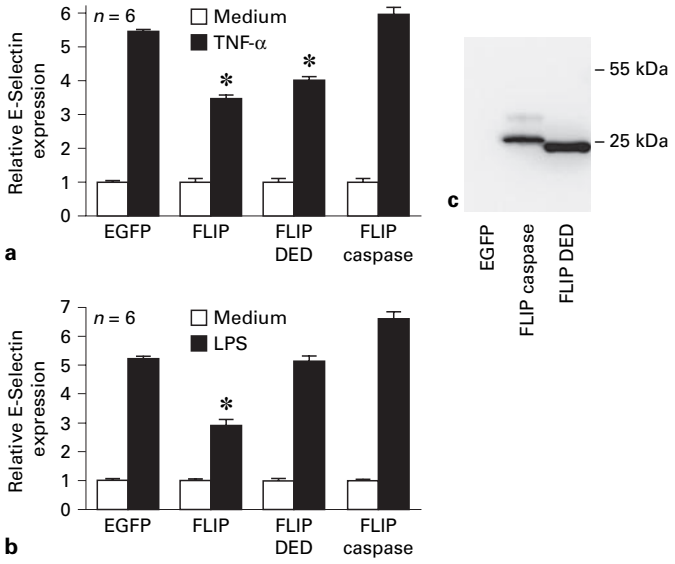

Fig. 3. Effect of bovine FLIP expression on TNF-α- and LPS-induced expression of the NF-κB-regulated gene product, E-selectin. Bovine aortic endothelial cells stably transfected with either EGFP-vector alone, full-length bovine FLIP, or the DED or caspase domains of bovine FLIP alone, were exposed for 8 h to medium, TNF-α (10 ng/ml) (**a**), or LPS (100 ng/ml) (**b**), and assayed by ELISA for E-selectin expression. Western blotting with an anti-FLAG antibody was used to confirm expression of the DED and caspase domains (**c**). Vertical bars represent mean (+S.E.) E-selectin expression relative to simultaneous medium-alone exposed cells. For each transfected endothelial cell line, the number of treatment replicates is indicated (n). * Significantly decreased compared to EGFP-vector transfected endothelial cells exposed to identical treatment.

(Fig. 3c). The DED region of FLIP alone was able to inhibit TNF-α -induced E-selectin expression to a comparable degree as the full-length FLIP protein (Fig. 3a). Interestingly, expression of the DED region alone had no inhibitory effect on LPS-induced EC activation (Fig. 3b). Further, the caspase domain appeared to lack any inhibitory effect on NF-κB activation elicited by either TNF-α or LPS (Fig. 3a and b). To our knowledge, this is the first report to identify the functional domain of FLIP that governs its ability to inhibit NF-κB-dependent gene expression induced by TNF-α. Further, the data presented here suggest that the ability of FLIP to downregulate LPS-induced NF-κB-dependent gene expression is dependent upon the presence of regions within both the amino- and carboxy termini of FLIP. Whether it is the DED and caspase domains that are required, or other unidentified functional domains, remains to be determined.

The finding that the FLIP DED region alone could inhibit TNF-α-, but not LPS-induced NF-κB-dependent gene expression is consistent with known protein-protein interactions between FLIP and proteins involved in TNF-α-induced NF-κB activation. FLIP has been shown to bind to TNF-receptor associated factors 1 and 2 (Kataoka et al., 2000), the latter of which promotes TNF-α-induced NF-κB activation (Rothe et al., 1995). In contrast, FLIP is unable to associate with TRAF-6 (Kataoka et al., 2000), a protein that promotes LPS-induced NF-κB activation (Lomaga et al., 1999). This suggests that FLIP can exert its inhibitory effect, perhaps through its DED, in a stimulus-specific manner at the level of TRAF. FLIP has also been shown to bind to NF-κB-inducing kinase (NIK) and IκB kinases (IKK) (Chaudhary et al., 2000), which promote both TNF-α- and LPS-induced NF-κB activation (O'Connell et al., 1998; Fischer et al., 1999). Thus, at the level of these proteins, FLIP could inhibit NF-κB activation induced by either LPS or TNF-α by sequestering NIK and/or IKK.

The conservation across species of FLIP's ability to inhibit apoptosis and downregulate NF-κB activity contrasts with recent findings on the lack of functional conservation of the FLIP binding protein, FADD (Szperka et al., 2005). Bovine FADD, which was recently cloned and sequenced, was demonstrated to retain its pro-apoptotic signaling capabilities similar to its human and murine counterparts. In contrast to its human homologue (Bannerman et al., 2002), however, bovine FADD lacks any NF-κB regulatory activity (Szperka et al., 2005). In the present report, we have established that bovine FLIP, similar to its human homologue (Bannerman et al., 2004), is able to both protect against LPS- and TNF-α-induced apoptosis and to downregulate NF-κB-dependent gene expression elicited by either agent.

In conclusion, the bovine FLIP cDNA was sequenced, its gene mapped, and the protein functionally characterized. The data presented here establish that bovine FLIP is capable of functioning as an anti-apoptotic signaling molecule that has the additional ability to moderate NF-κB-dependent gene expression. Further studies are required to investigate the mechanism by which FLIP and/or its binding domains downregulate NF-κB activation and the influence that changes in FLIP expression have on the outcome of diseases such as Gram-negative mastitis, where much of the underlying pathogenesis is mediated by LPS and TNF-α. Further, because of its chromosomal localization near a putative QTL for functional herd life, variation in the bovine FLIP gene and its association with this economically important trait warrants additional study.

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